PLASMID AND PHAGE VECTORS

- **A vector is used to amplify a single molecule of DNA into many copies.**

- **A DNA fragment must be inserted into a cloning vector.**

- **A cloning vector is a DNA molecule that has an origin of replication and is capable of replicating in a bacterial cell.**

General Features of Vectors

➢Vectors must be able to replicate autonomously in the host cell and must have at least one usable restriction enzyme site for the introduction of foreign DNA.

➢In addition, a vector should also have:
➢• selectable markers (to select for cells that have taken up the vector DNA);

➢• a range of restriction enzyme sites available for cloning,

➢• insertional inactivation indicator genes (i.e., genes that are insertionally
➢disrupted by the cloning process to indicate that cloning has occurred);
➢• a promoter (preferably a strong one) upstream of the cloning site;

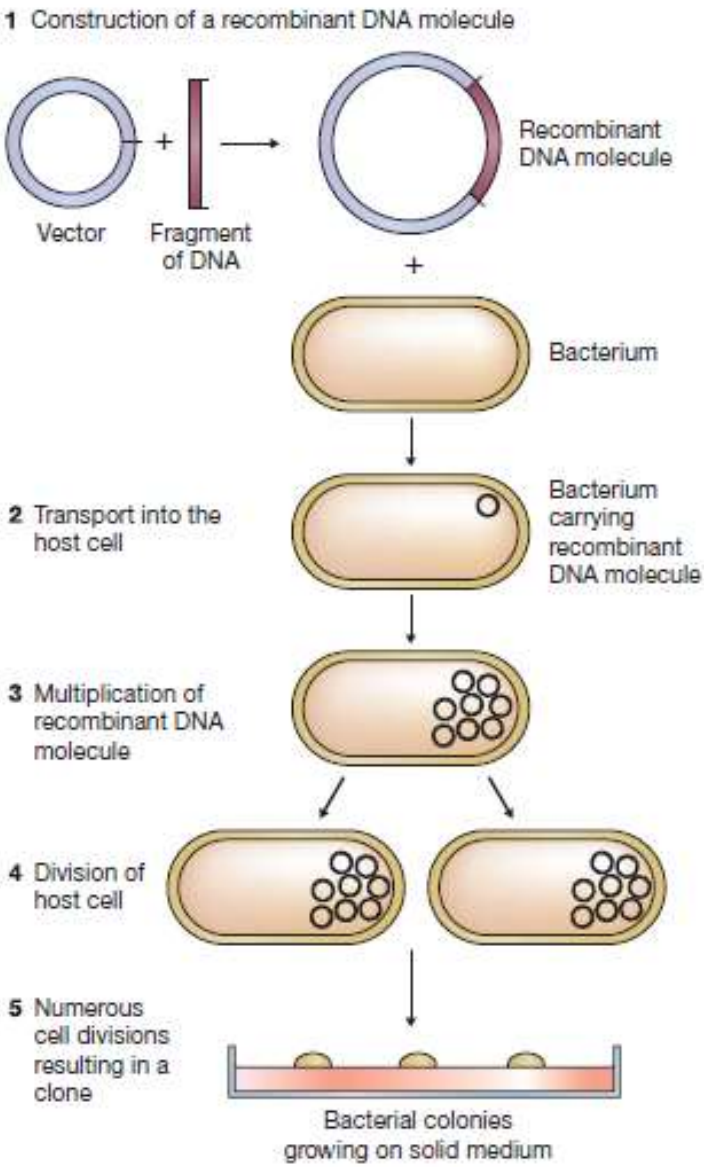
➢• a terminator (strong enough to match the strength of the promoter)
➢downstream of the cloning site (this makes the vector an expression vector);

➢• an origin of replication

Plasmid

- Plasmids are circular, double-stranded DNA molecules that exist in bacteria and in the nuclei of some eukaryotic cells.
- They can replicate independently of the host cell.
- The size of plasmids ranges from a few kb to near 100 kb
- They can hold up to 10 kb fragments

Basic steps in gene cloning

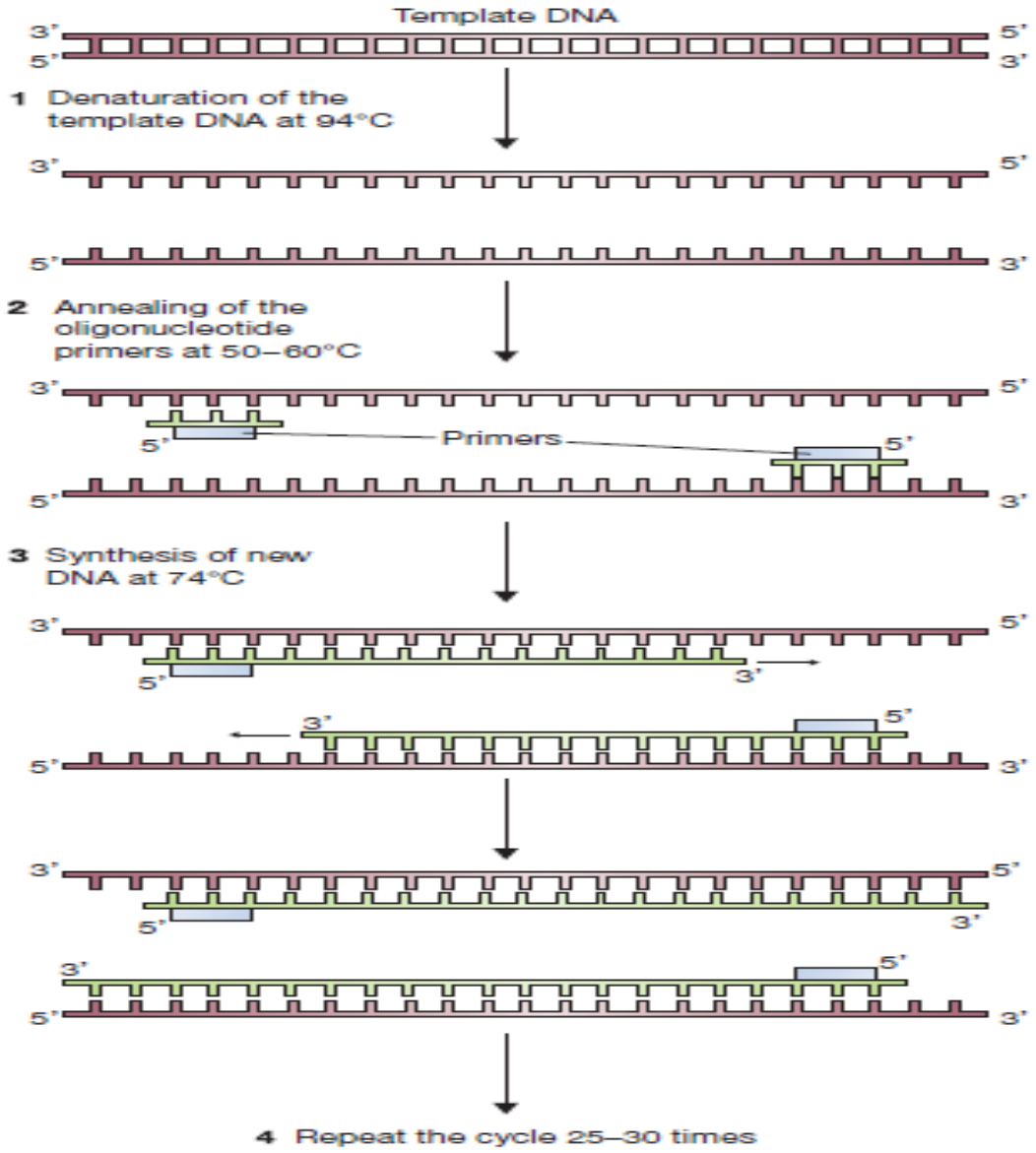


1.3 What is gene cloning?

What exactly is gene cloning? The easiest way to answer this question is to follow through the steps in a gene cloning experiment (Figure 1.1):

1. A fragment of DNA, containing the gene to be cloned, is inserted into a circular DNA molecule called a **vector**, to produce a **recombinant DNA molecule**.
2. The vector transports the gene into a host cell, which is usually a bacterium, although other types of living cell can be used.
3. Within the host cell the vector multiplies, producing numerous identical copies, not only of itself but also of the gene that it carries.
4. When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place.
5. After a large number of cell divisions, a colony, or **clone**, of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule; the gene carried by the recombinant molecule is now said to be cloned.

PCR


Template DNA
3'
5'
5'
3'
1 Denaturation of the template DNA at 94°C
2 Annealing of the oligonucleotide primers at 50–60°C
Primers
3 Synthesis of new DNA at 74°C
4 Repeat the cycle 25–30 times

Summary

1. The mixture is heated to 94°C, at which temperature the hydrogen bonds that hold together the two strands of the double-stranded DNA molecule are broken, causing the molecule to denature.
2. The mixture is cooled down to 50–60°C. The two strands of each molecule could join back together at this temperature, but most do not because the mixture contains a large excess of short DNA molecules, called oligonucleotides or primers, which anneal to the DNA molecules at specific positions.
3. The temperature is raised to 74°C. This is a good working temperature for the *Taq* DNA polymerase that is present in the mixture. We will learn more about DNA polymerases on p. 48. All we need to understand at this stage is that the *Taq* DNA polymerase attaches to one end of each primer and synthesizes new strands of DNA, complementary to the template DNA molecules, during this step of the PCR. Now we have four stands of DNA instead of the two that there were to start with.
4. The temperature is increased back to 94°C. The double-stranded DNA molecules, each of which consists of one strand of the original molecule and one new strand of DNA, denature into single strands. This begins a second cycle of denaturation–annealing–synthesis, at the end of which there are eight DNA strands. By repeating the cycle 30 times the double-stranded molecule that we began with is converted into over 130 million new double-stranded molecules, each one a copy of the region of the starting molecule delineated by the annealing sites of the two primers.

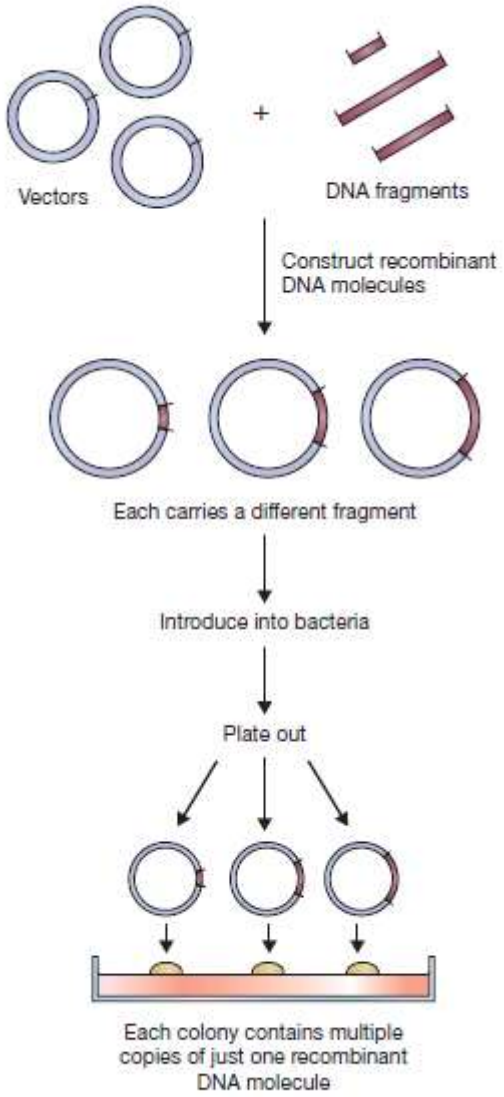


Figure 1.3

Cloning allows individual fragments of DNA to be purified.

Basic biology of a plasmid vector

Figure 2.1

Plasmids: independent genetic elements found in bacterial cells.

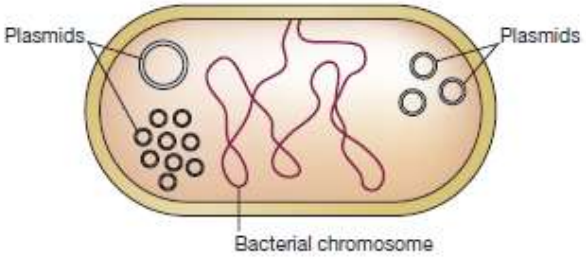


Figure 2.2

The use of antibiotic resistance as a selectable marker for a plasmid. RP4 (top) carries genes for resistance to ampicillin, tetracycline and kanamycin. Only those *E. coli* cells that contain RP4 (or a related plasmid) are able to survive and grow in a medium that contains toxic amounts of one or more of these antibiotics.

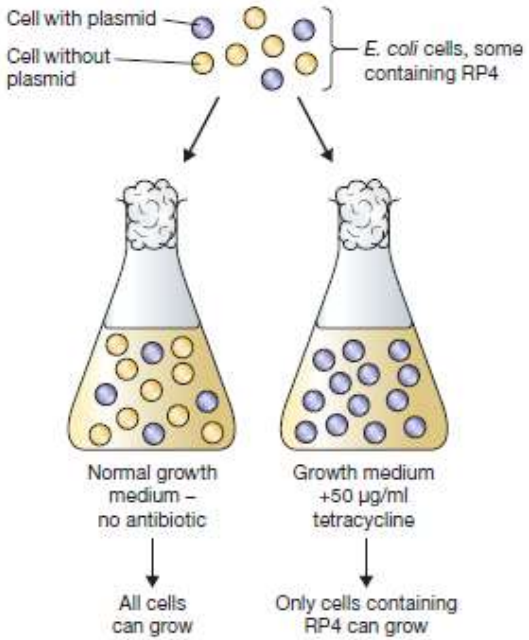
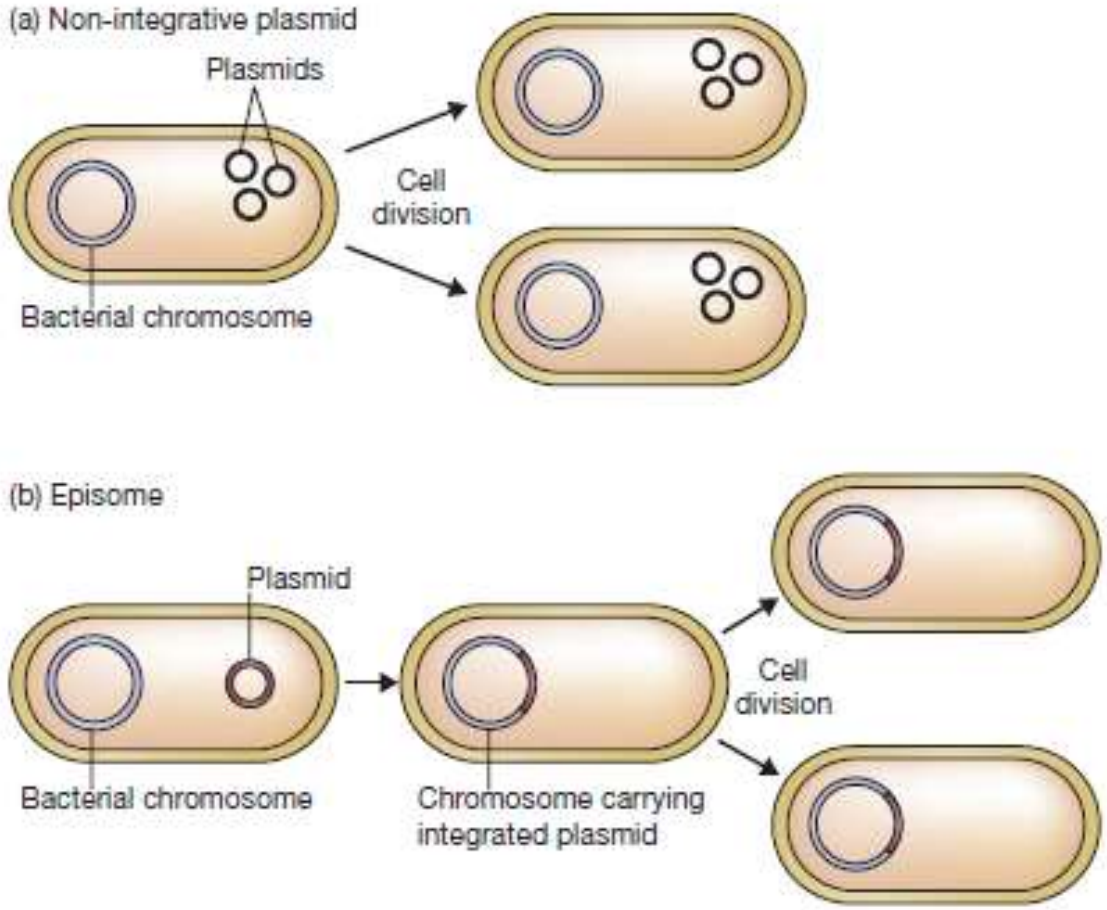


Figure 2.3

Replication strategies for (a) a non-integrative plasmid, and (b) an episome.



Conjugative plasmid

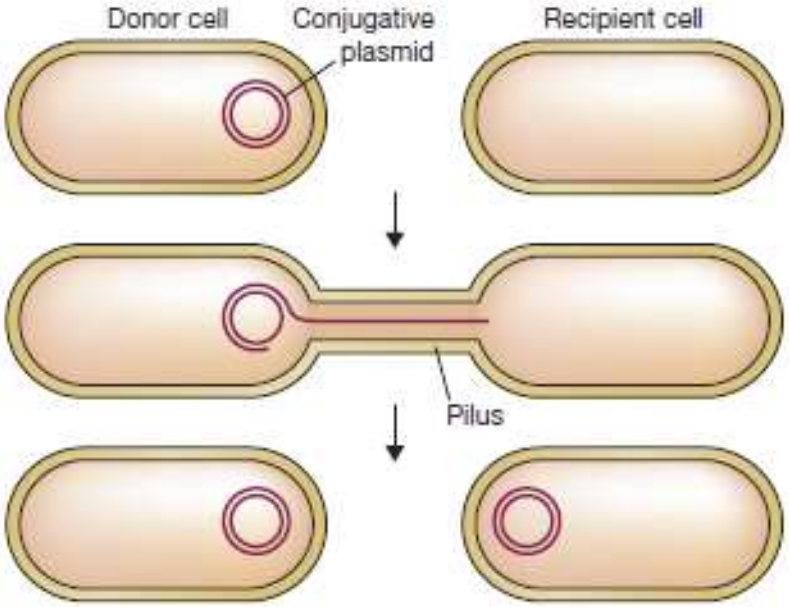
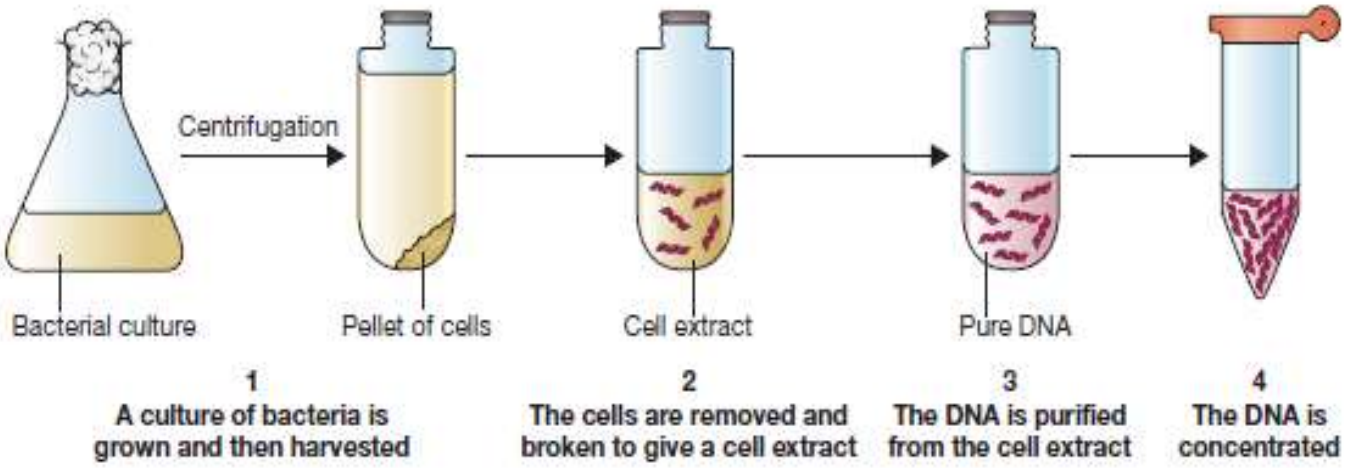


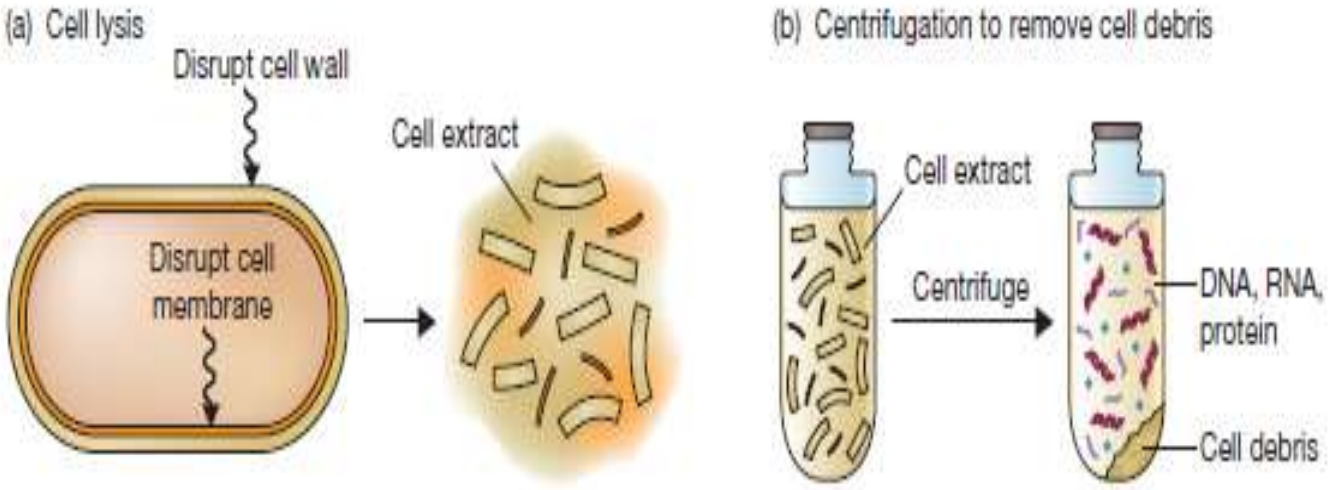
Figure 2.4

Plasmid transfer by conjugation between bacterial cells. The donor and recipient cells attach to each other by a pilus, a hollow appendage present on the surface of the donor cell. A copy of the plasmid is then passed to the recipient cell. Transfer is thought to occur through the pilus, but this has not been proven and transfer by some other means (e.g. directly across the bacterial cell walls) remains a possibility.

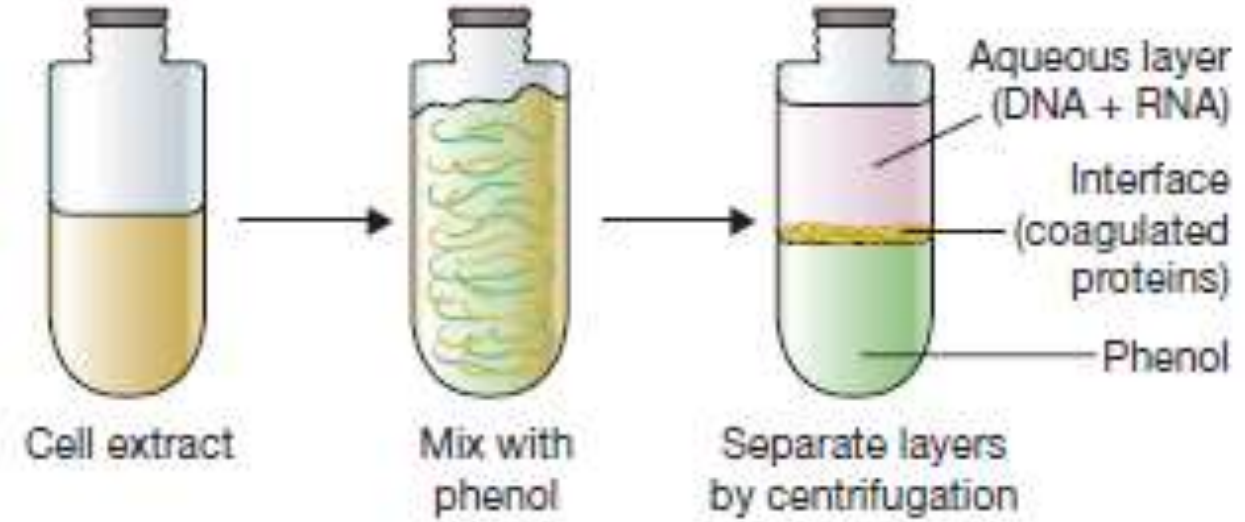
The basic steps in preparation of total cell DNA from a culture of bacteria



Preparation of a cell extract



Removal of protein contaminants by phenol extraction.



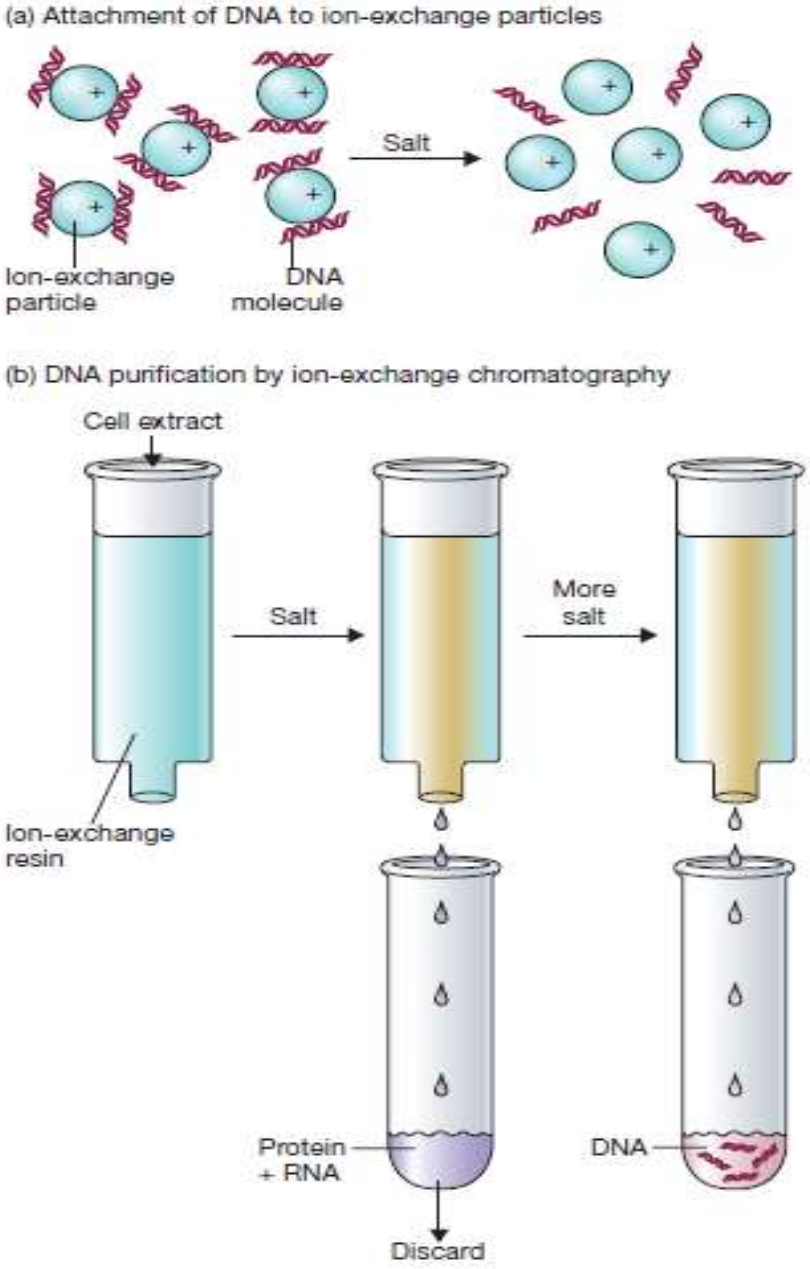


Figure 3.7

DNA purification by ion-exchange chromatography. (a) Attachment of DNA to ion-exchange particles. (b) DNA is purified by column chromatography. The solutions passing through the column can be collected by gravity flow or by the **spin column** method, in which the column is placed in a low-speed centrifuge.

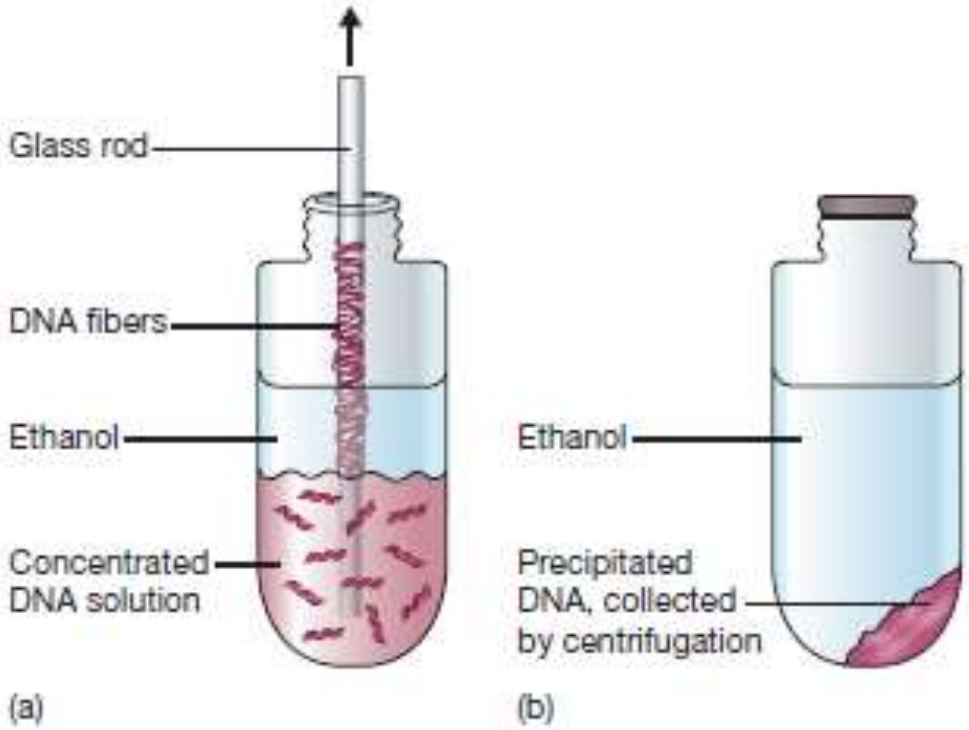


Figure 3.8

Collecting DNA by ethanol precipitation. (a) Absolute ethanol is layered on top of a concentrated solution of DNA. Fibers of DNA can be withdrawn with a glass rod. (b) For less concentrated solutions ethanol is added (at a ratio of 2.5 volumes of absolute ethanol to 1 volume of DNA solution) and precipitated DNA collected by centrifugation.

Preparation of a cleared lysate.

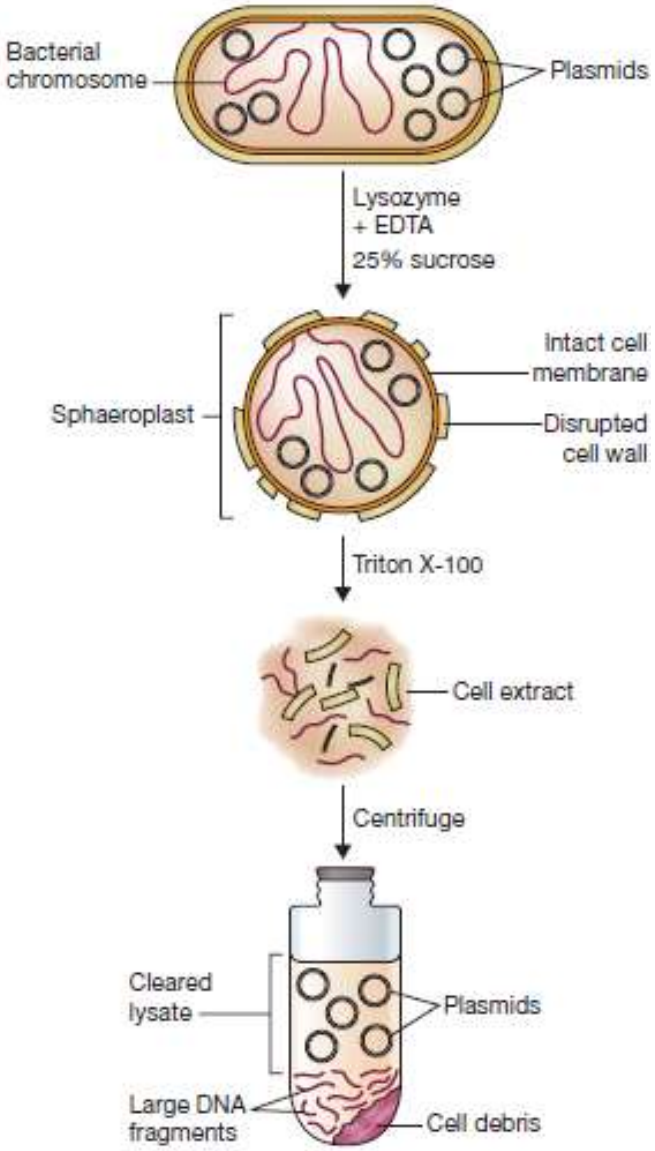
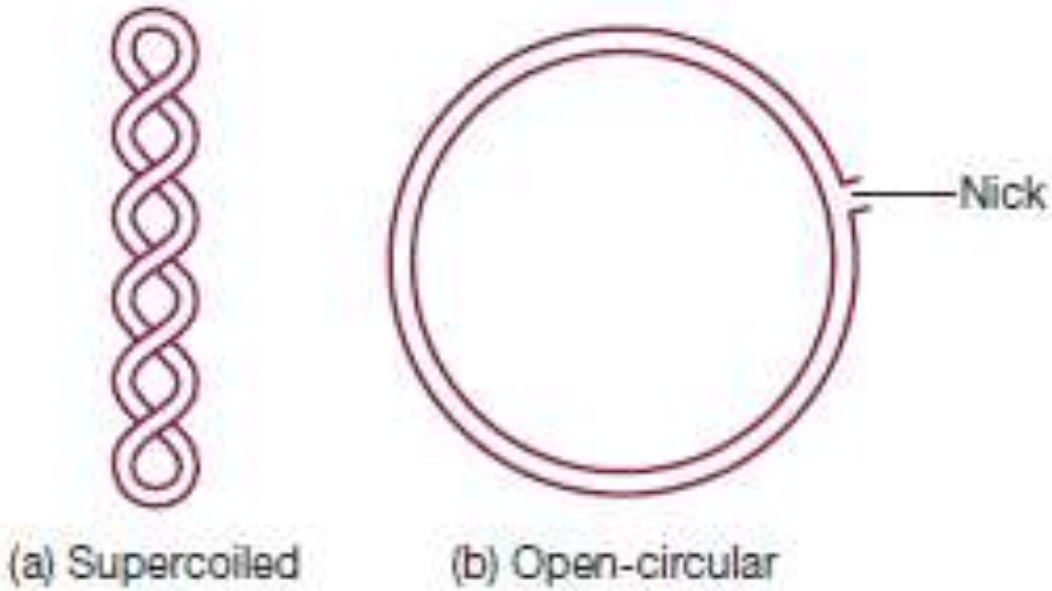
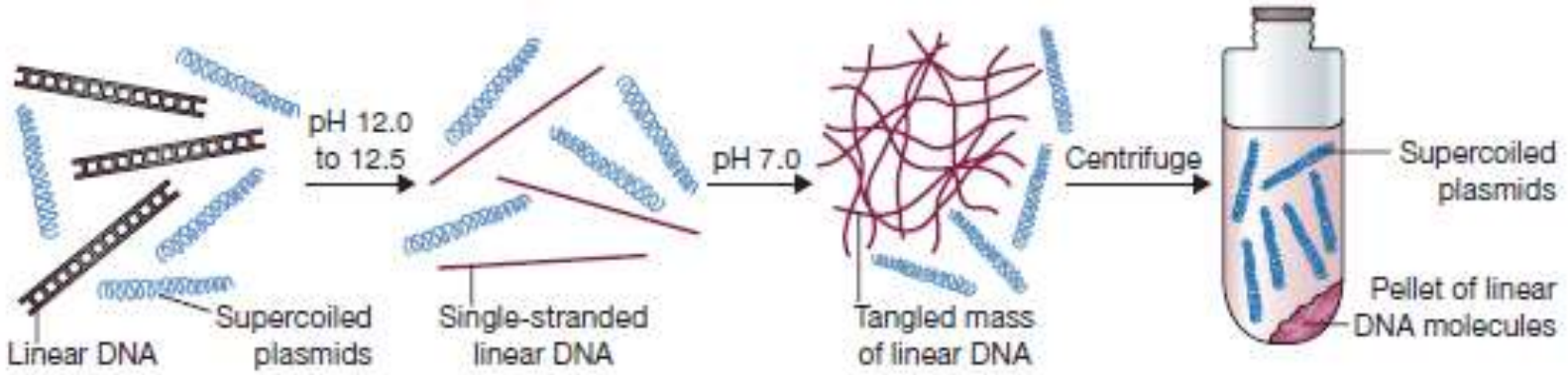


Figure 3.12

Two conformations of circular double-stranded DNA: (a) supercoiled—both strands are intact; (b) open-circular —one or both strands are nicked.

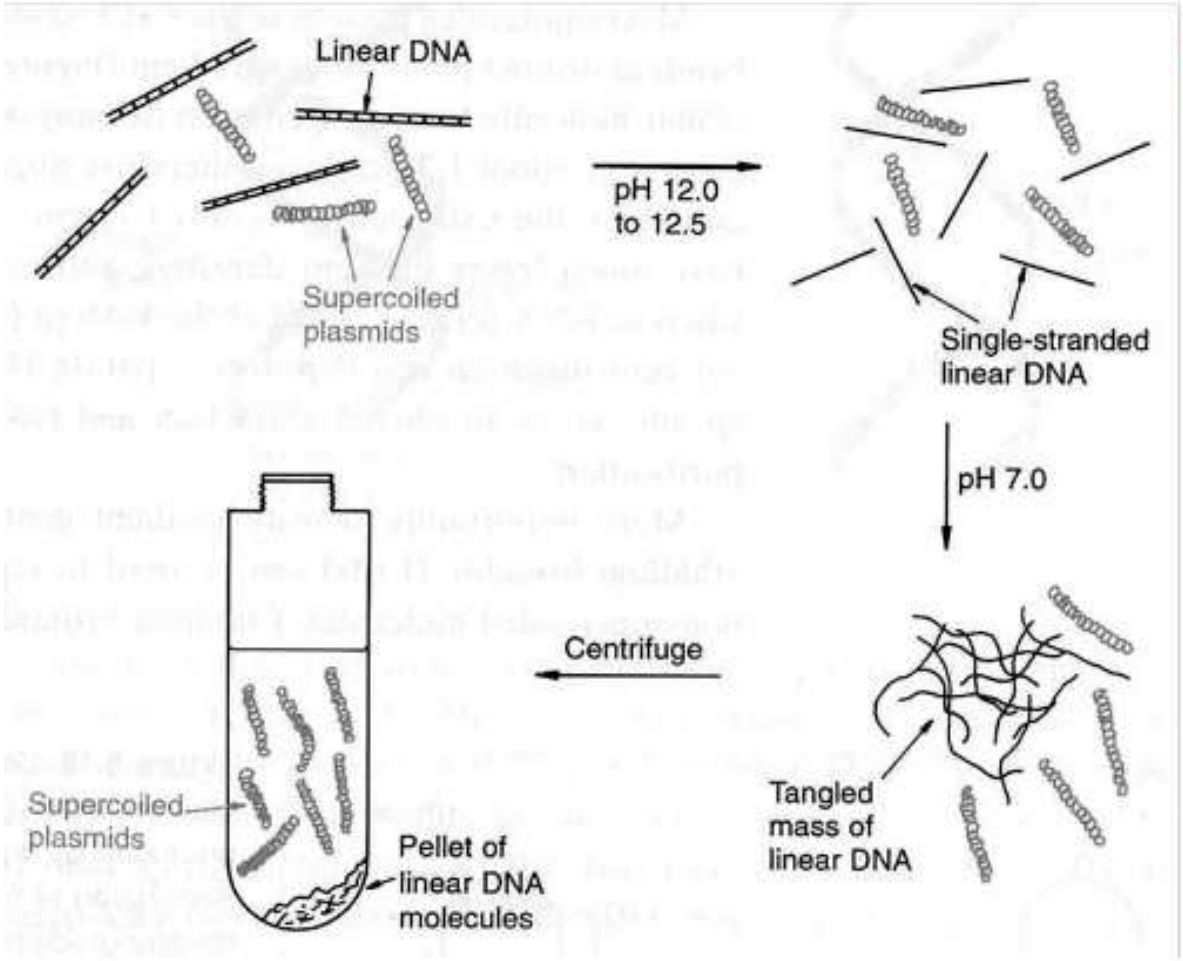


Plasmid purification by the alkaline denaturation method



Plasmid Isolation

Plasmid purification: alkaline lysis



Alkaline conditions denature DNA

Neutralize: genomic DNA can't renature (plasmids CAN because they never fully separate)

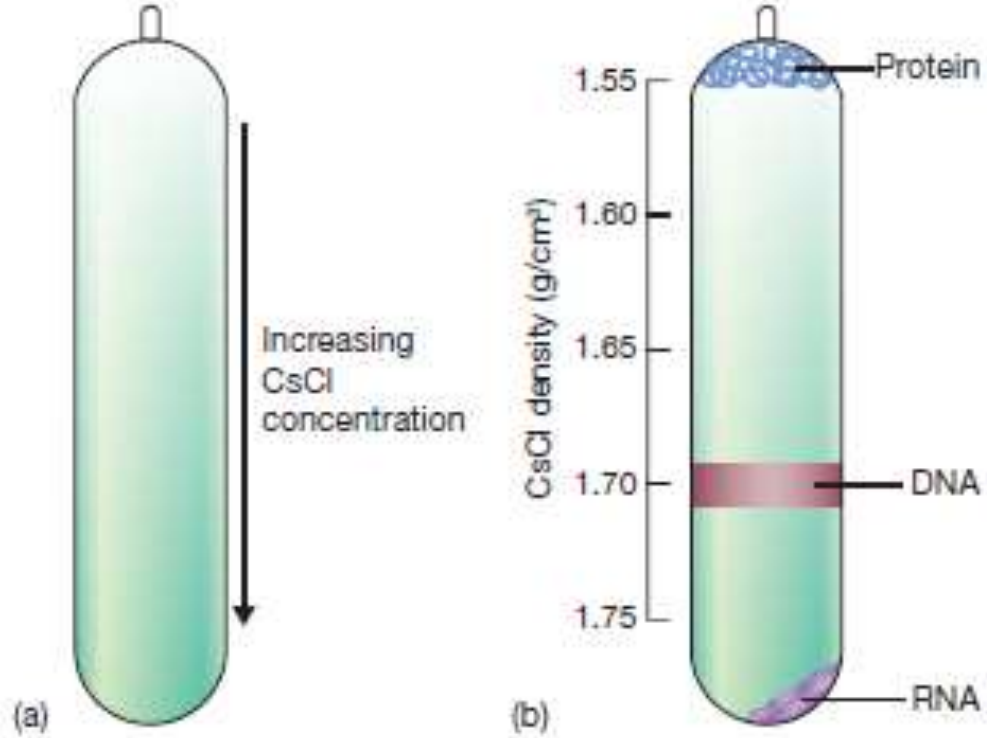
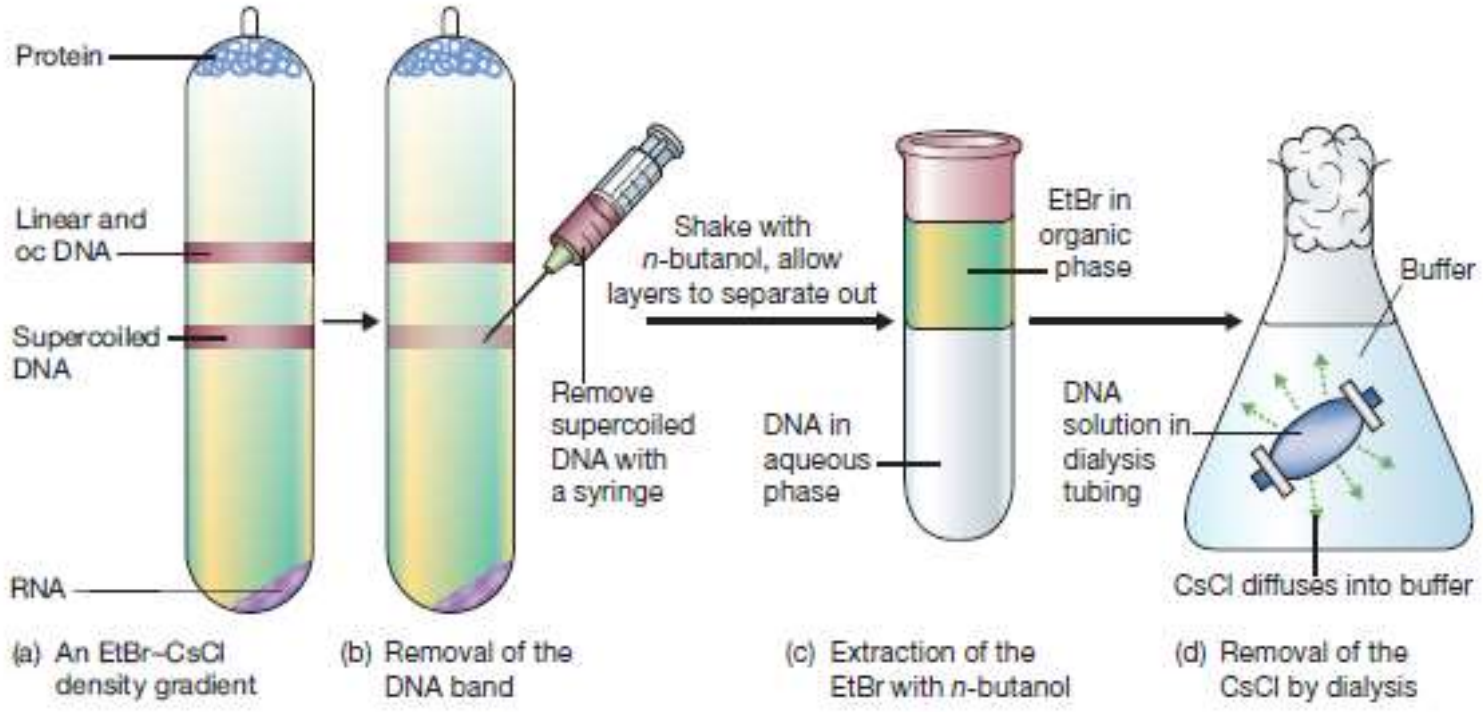


Figure 3.14

Caesium chloride density gradient centrifugation. (a) A CsCl density gradient produced by high speed centrifugation. (b) Separation of protein, DNA, and RNA in a density gradient.

Purification of plasmid DNA by EtBr–CsCl density gradient centrifugation



Manipulation of Purified DNA

DNA manipulating enzyme

DNA manipulative enzymes can be grouped into four broad classes, depending on the type of reaction that they catalyze:

- Nucleases are enzymes that cut, shorten, or degrade nucleic acid molecules.
- Ligases join nucleic acid molecules together.
- Polymerases make copies of molecules.
- Modifying enzymes remove or add chemical groups.

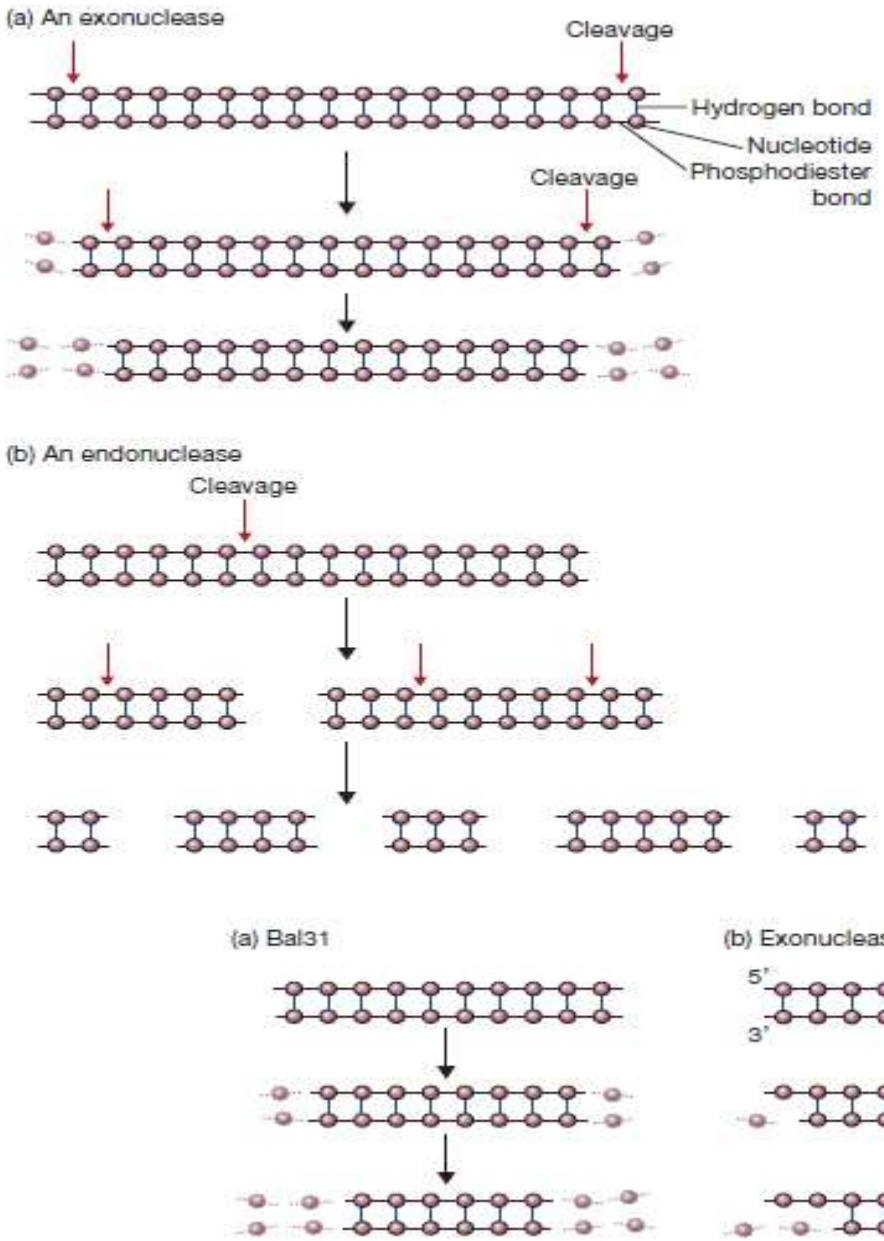


Figure 4.1

The reactions catalyzed by the two different kinds of nuclease. (a) An exonuclease, which removes nucleotides from the end of a DNA molecule. (b) An endonuclease, which breaks internal phosphodiester bonds.

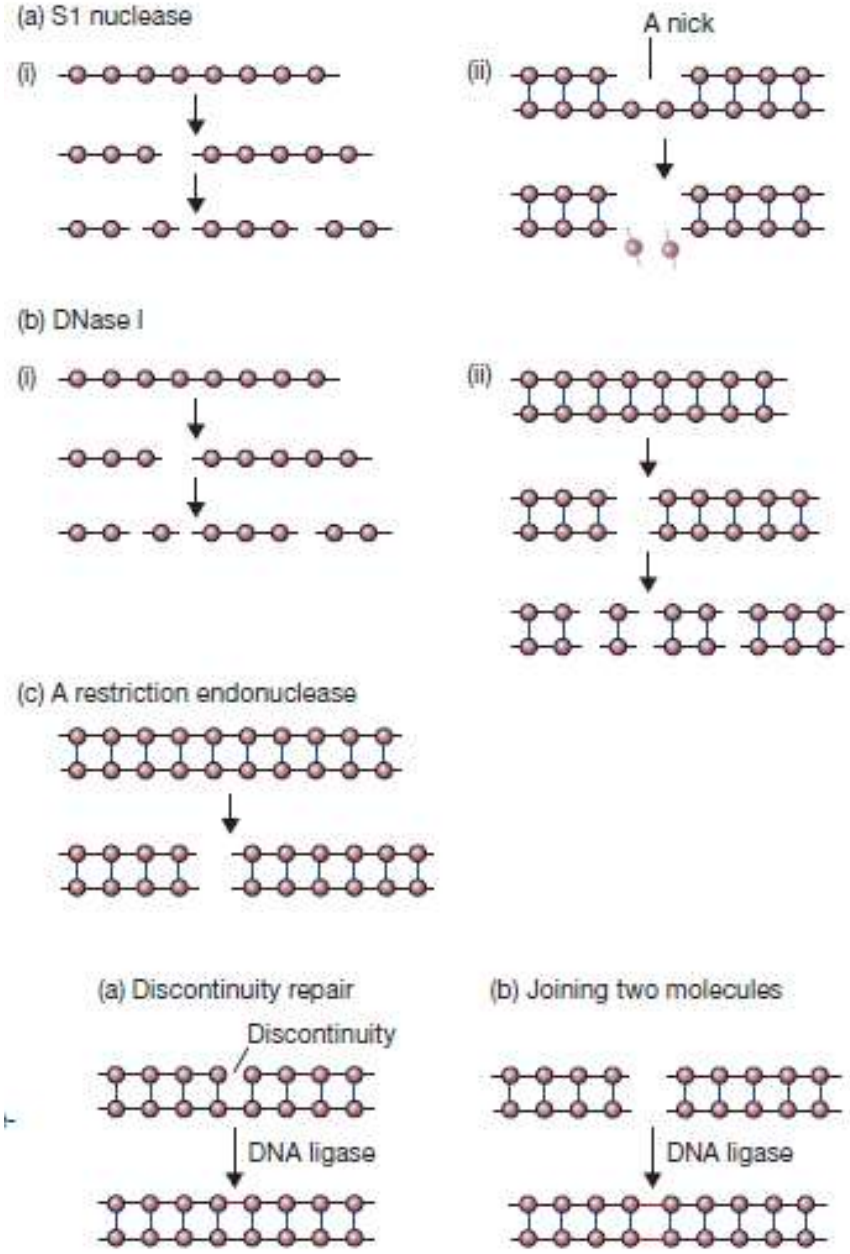


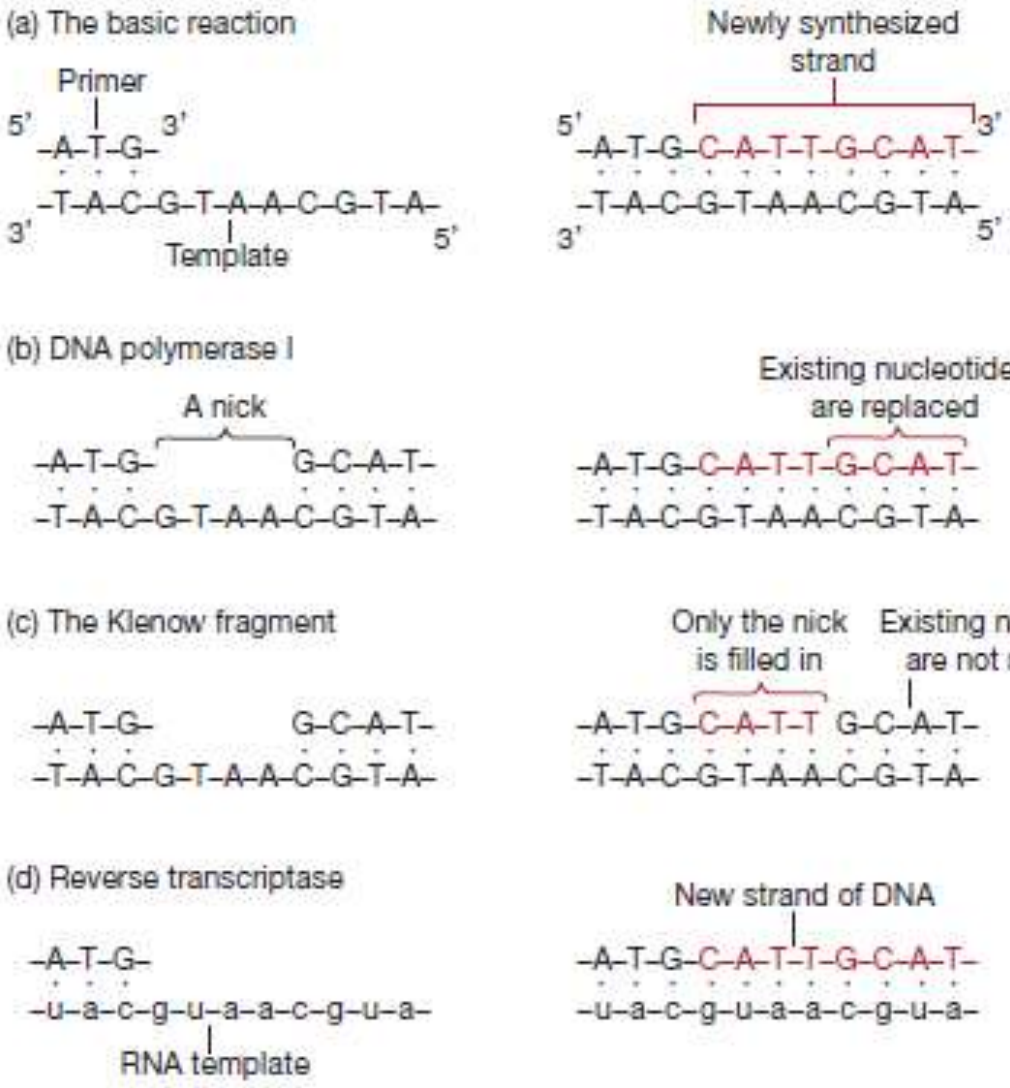
Figure 4.3

The reactions catalyzed by different types of endonuclease. (a) S1 nuclease, which cleaves only single-stranded DNA, including single-stranded nicks in mainly double-stranded molecules. (b) DNase I, which cleaves both single- and double-stranded DNA. (c) A restriction endonuclease, which cleaves double-stranded DNA, but only at a limited number of sites.

Figure 4.4

The two reactions catalyzed by DNA ligase. (a) Repair of a discontinuity—a missing phosphodiester bond in one strand of a double-stranded molecule. (b) Joining two molecules together.

The reactions catalyzed by DNA polymerases



The reactions catalyzed by DNA polymerases

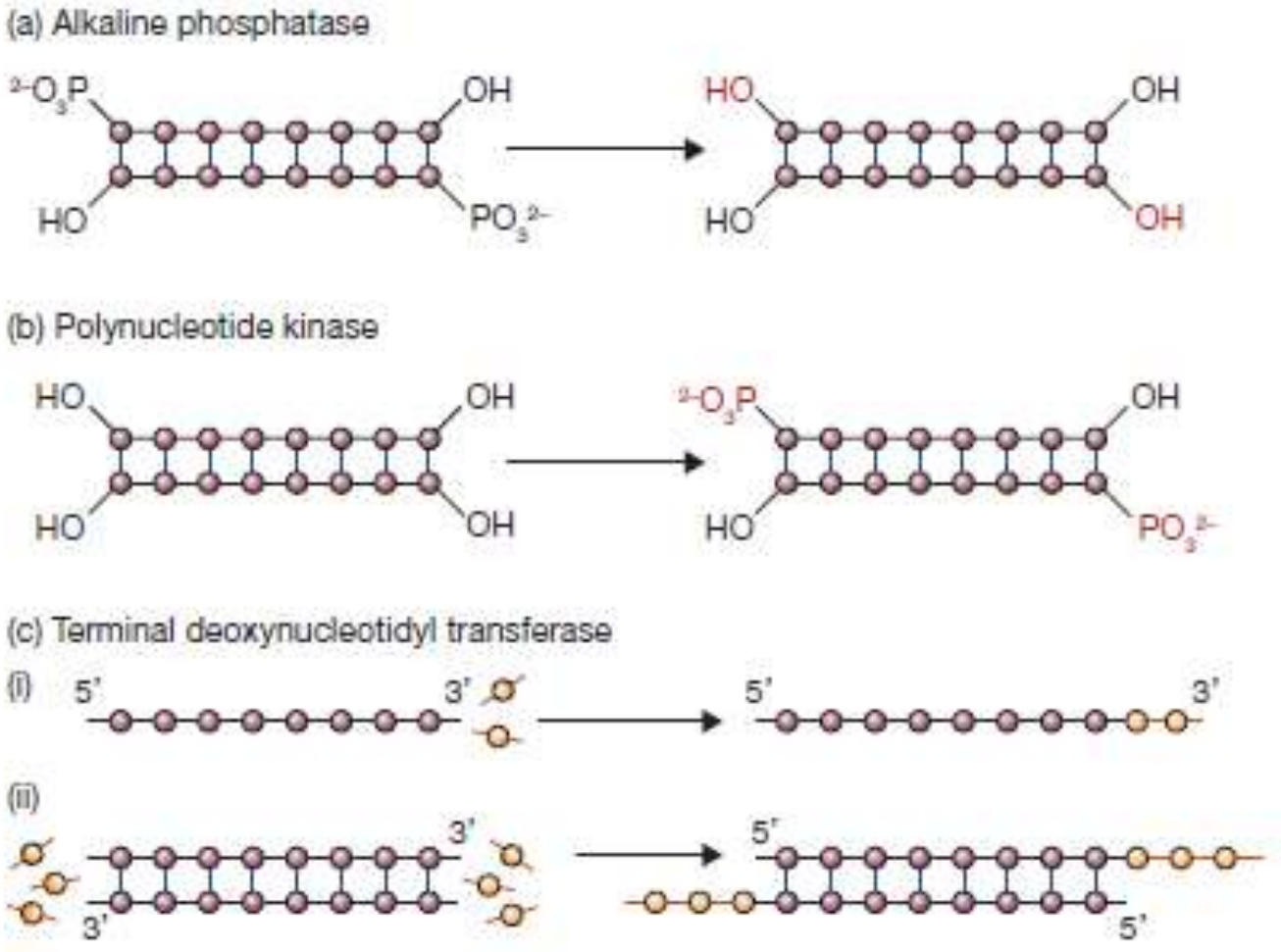
- **(a) The basic reaction: a new DNA strand is synthesized in the**
- **5′ to 3′ direction.**

- **(b) DNA polymerase I, which initially fills in nicks but then continues to synthesize a new strand, degrading the existing one as it proceeds.**

- **(c) The Klenow fragment, which only fills in nicks.**

- **(d) Reverse transcriptase, which uses a template of RNA.**

The reactions catalyzed by DNA modifying enzymes



The reactions catalyzed by DNA modifying enzymes

The reactions catalyzed by DNA modifying enzymes.

(a) Alkaline phosphatase, which removes 5′-phosphate groups.

(b) Polynucleotide kinase, which attaches 5′-phosphate groups.

(c) Terminal deoxynucleotidyl transferase, which attaches deoxyribonucleotides to the 3′ termini of polynucleotides in either (i) single-stranded or (ii) double-stranded molecules